A 31-RESIDUE TRYPTIC PEPTIDE FROM THE ACTIVE SITE OF THE [Ca^{++}]-TRANSPORTING ADENOSINE TRIPHOSPHATASE OF RABBIT SARCOPLASMIC RETICULUM

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Received 20 January 1976

1. Introduction

The calcium-transporting adenosine triphosphatase (ATPase) of rabbit sarcoplasmic reticulum is phosphorylated at an aspartyl residue during the enzymic hydrolysis of ATP [1]. The lability of the acyl phosphate bond prevents the ready isolation of phosphopeptides from the phosphoenzyme. By studying the chemical and electrophoretic properties of [^{32}P] phosphopeptides produced by various proteases, Bastide et al. [2] showed that a probable active-site tripeptide sequence was (Ser or Thr)–Asp–Lys. They also had evidence for the presence of a cysteine residue in some of their larger peptides. Since we had already isolated and sequenced a number of cyteinc peptides from a tryptic digest of carboxymethylated ATPase we decided to isolate a tryptic active site peptide for comparison of its composition with that of the known peptides.

We describe here the isolation of this peptide, containing 31 residues, all but six of which have been placed in sequence.

2. Methods

Sarcoplasmic reticulum vesicles were isolated by a method similar to that of Martonosi [3]. All operations were at 0–5°C and 1 mM 2-mercaptoethanol was included in all buffers. $H_3{}^{32}PO_4$ was obtained from the Radiochemical Centre, Amersham, Bucks, UK, and [γ-[^{32}P]ATP was prepared by the method of Glynn and Chappell [4]. Radioactivity was determined by liquid scintillation counting in toluene/2-methoxyethanol (3:1, v/v) containing 4.9 g/litre of 2,5-bis-(5-t-butylbenzoxazol-2-yl) thiophene. Radioautographs of electrophoretograms were prepared by overnight contact of dried papers with Kodak Kodirex AutoProcess X-ray film.

The phosphorylated protein was prepared at pH 7.0, by the method of Degani and Boyer [1]. The precipitated protein (300 mg) was homogenized in a glass homogenizer in water (5 ml), and the pH was adjusted to 7.0–7.5 with 1 M Tris solution. Trypsin (30 mg; Worthington, 2 × crystallised), in 0.5 ml of water, was added and homogenisation was continued for 10 min at 20°C with addition of 1 M Tris buffer (adjusted to pH 8.5 with HCl) to maintain pH 7–7.5. Most of the precipitated protein was solubilized by this procedure. Iodoacetamide (40 mg) was added in 1 ml of water, and carboxamidomethylation was allowed to proceed at 20°C for 10 min. 20% (w/v) Trichloroacetic acid (5 ml) was added, and the precipitate was removed by centrifugation at 4000 *g* for 5 min. The phosphopeptide was purified from the supernatant as described in the Results section.

Peptic phosphopeptides were isolated in a similar way after digestion of the perchloric acid precipitated protein with pepsin (150 mg) in 20 mM HCl for 35 min. The digested protein was not treated with iodoacetamide, and trichloroacetic acid was not

Abbreviation: Dansyl, 5-dimethylaminonaphthalene-1-sulphonyl.
Enzymes: ATPase (EC 3.6.1.3); Trypsin (EC 3.4.21.4); Pepsin (EC 3.4.23.1).

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added before application of the soluble part of the peptic digest to the Sephadex G-25 column.

Carboxymethylcysteine-containing tryptic peptides were isolated from reduced, [^{14}C]carboxymethylated ATPase. A full description of this work is in preparation, but we include here a brief outline of the procedures used. ATPase was purified by the method of MacLennan [5], with the inclusion of 1 mM *N*-acetylcysteine in the extraction buffer. Several approaches were used for carboxymethylation with [^{14}C]iodoacetate, delipidation and tryptic digestion of the protein. Carboxymethylation of the ATPase protein after reduction with dithiothreitol in 6 M guanidinium chloride containing 5% (w/v) sodium taurodeoxycholate gave the most efficient labelling. The reaction mixture was dialysed and lipid was separated from the protein on a column of Sepharose 6-B in 1% (w/v) sodium dodecyl sulphate. Dodecyl sulphate was removed on a column of BioRad AG 1x2 anion exchange resin in 8 M urea, and the protein solution was dialysed against water.

The protein was digested with trypsin at pH 8, and the digest was resolved into several fractions on a column of Sephadex G-50. The radioactive peptides were further purified by chromatography on DEAE-cellulose, SE-Sephadex, paper electrophoresis and paper chromatography. The amino acid sequences of pure radioactive peptides were determined.

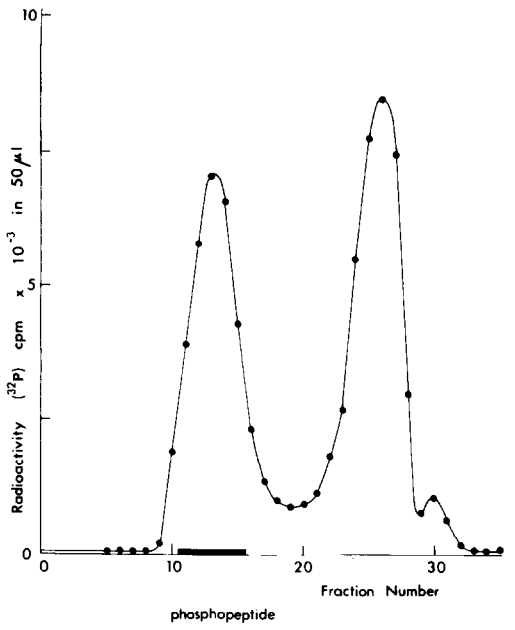

Fig.1. Elution of tryptic [^{32}P]phosphopeptides from a Sephadex G-25 (superfine) column (26 × 400 mm) in 50 mM acetic acid. The trichloroacetic acid supernatant of the tryptic digest of phosphorylated ATPase was applied in 18 ml. 30 ml of eluate was collected, followed by fractions of 5.5 ml, at a flow rate of 2 ml/min, at 25°C. Fractions were combined as indicated by the bar. The second peak was mainly orthophosphate.

Results

The tryptic digest was applied to a Sephadex G-25 column eluted as shown in fig.1. The phosphopeptide peak was applied directly to a column of Sulfoethyl Sephadex G-25 and eluted as described in the legend to fig.2. Some radioactive material which passed straight through the column was shown to be

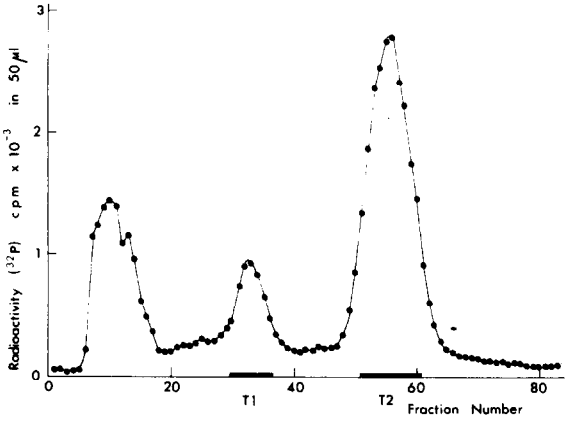

Fig.2. Elution of tryptic [^{32}P]phosphopeptides from a column of Sulfoethyl Sephadex C-25 (13 × 200 mm). The column was equilibrated with 50 mM pyridine, 100 mM acetic acid, 400 mM formic acid, pH 2.7. The phosphopeptides were applied in 27 ml of 50 mM acetic acid, and were eluted with 2 ml of pH 2.7 buffer followed by a gradient formed from 200 ml of the pH 2.7 buffer and 200 ml of pH 4.1 buffer, containing pyridine (250 mM), acetic acid (250 mM) and formic acid (225 mM). Fractions of 2.6 ml were collected at 1.3 ml/min at 25°C. Fractions were combined as shown to give peptides T1 and T2, which were purified by diagonal paper electrophoresis as described in the text.

orthophosphate by paper electrophoresis and radioautography.

The phosphopeptides were purified by electrophoresis on Whatman no. 3 MM paper at pH 2, in acetic acid/formic acid/water (8:2:90, v/v/v) [6], for 2 h at 100 V/cm, using a Shandon flat-bed apparatus with water-cooled plates. After drying at room temperature, the paper was sprayed lightly with 5% (v/v) aqueous *N*-ethylmorpholine (re-distilled from ninhydrin) to hydrolyse the acyl-phosphate bond. After slow drying at room temperature, the paper was radioautographed. Phosphopeptide T1 had mobility, relative to that of ε-dinitrophenyl-lysine, 0.63, and phosphopeptide T2 had mobility 0.71.

The dephosphorylated peptides were eluted with 5% (v/v) formic acid, and electrophoresis was repeated at pH 2. Peptides were located with 0.01% (w/v) flurorescamine (Roche Diagnostics, Nutley, NJ, USA)

Table 1
Amino acid analyses of tryptic and peptic peptides from the [Ca^{++}]ATPase of rabbit sacroplasmic reticulum

	Peptide T1a[a]	Peptide T2a	[^{14}C]carboxy-methylated peptide T3	[^{14}C]carboxy-methylated peptide T4	Oxidised peptic peptide
$CysCH_2COOH$	–[b]	2[c,d]	3.0	2[c]	–
Asp	+	2[c]	2.0	1[c]	1.4
Thr	+[e]	5.0	5.0	2.0	2.0
Ser	+[e]	3.7	4.1	3.4	1.5
Glu	++	2.2	2.0	1.3	0.2
Pro	–[e]	0.9	0.7	0.9	–
Gly	++	2.2	2.1	1.1	1.2
Ala	+	0.2	–	–	–
1/2-Cys	–[b]	0.6[d]	–	–	1.0[f]
Val	++	2.2[g]	2.4[g]	1.7[g]	0.3[g]
Met	–	0.7	0.7	–	–[f]
Ile	+	0.6[g]	0.7[g]	0.6[g]	0.9
Leu	++	2.5	3.0	2.0	0.9
Tyr	–	–	–	–	–
Phe	–	–	–	–	–
His	–	–	–	–	–
Lys	+	1.4	1.8	1.0	1.0
Arg	–	–	–	–	–

[a] Qualitative analysis by the dansyl technique.
[b] Not readily determined by this technique.
[c] $CysCH_2COOH$ was not completely resolved from Asp in these analyses, and the values are approximate.
[d] The carboxamidomethylation was presumably incomplete, resulting in a low value for $CysCH_2COOH$ and the appearance of some cystine.
[e] These residues are underestimated by this method.
[f] Determined as cysteic acid or methionine sulphone.
[g] A Val–Ile bond is present, which is not completely hydrolysed after 20 h, and the yields of these residues are therefore low.

in acetone containing 1% (v/v) pyridine. Peptide T1 gave two bands, with mobility relative to ε-dinitrophenyl-lysine 0.93 (T1a) and 0.65 (T1b), while peptide T2 gave a broad band, with mobility 0.74–0.93. Half of the latter band, with mobility 0.84–0.93 (T2a) and band T1a were eluted with 5% (v/v) formic acid solution. These peptides, T1a and T2a, the mobilities of which had increased following dephosphorylation, were analysed, after hydrolysis with 6 M HCl containing 0.1% phenol, for 20 h at 110°C.

The amino acid analysis of peptide T1a, which was isolated in low yield (about 5 nmol), was performed qualitatively by dansylation of the peptide hydrolysate and chromatography of the dansyl-amino acids on polyamide layers in the solvent systems described by Hartley [7]. The amino acid analysis of peptide T2a, which was obtained in a yield of 18 nmol (about 1% molar yield from the ATPase), was determined on a BioCal amino acid analyser, and is given in table 1, in comparison with the analyses of two peptides (T3 and T4) isolated from the tryptic digest of reduced and [^{14}C]carboxymethylated ATPase. None of the 17 other cysteine-containing tryptic peptides, most of which have been characterised, bears any resemblance to peptides T1 and T2. The partial sequences of peptides T3 and T4 have been determined (G. Allen and N. M. Green, manuscript in preparation), using thermolysin fragments and the dansyl-Edman technique [8], and the results are presented in fig.3. Peptide T2a had N-terminal serine, as did peptides T3 and T4, as shown by the dansyl method [9].

Peptic phosphopeptides were isolated in a similar way, but the final purification step was a diagonal procedure involving oxidation of the phosphopeptides, after the first pH 2 electrophoresis, on paper with performic acid vapour [10] followed by repeated electrophoresis at pH 2, and the phosphopeptides were eluted intact from the paper. Amino acid analysis and N-terminal determination of the peptic peptide thus obtained (in 3% molar yield) identify it as a mixture of closely related peptides, due to alternative peptic cleavages, from the central portion of peptide T3 (table 1 and fig.3). Preliminary work on the sequences of the peptic phosphopeptides have shown that the structure is consistent with this part of peptide T3. Work is in progress to complete the sequence of this region of the polypeptide chain of the ATPase.

Discussion

The methods used here were chosen for rapidity, for use of low pH, where the phosphopeptide is least labile [2] and for use of 'diagonal' procedures for the final step of purification. The only change in

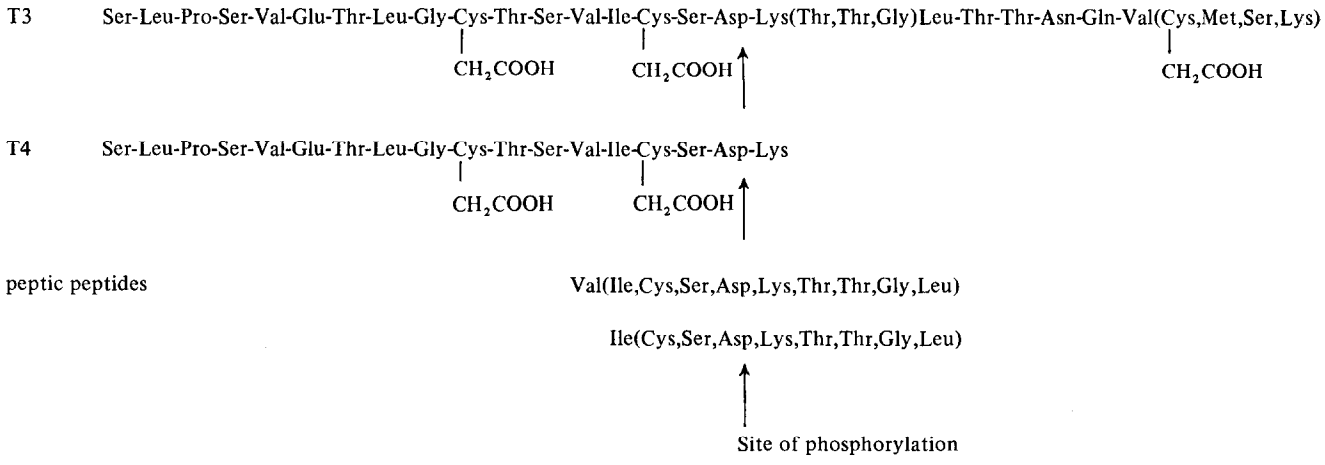

Fig.3. Sequence information obtained for peptides T3 and T4. Asparagine/aspartic acid and glutamine/glutamic acid ambiguities were resolved by the determination of electrophoretic mobilities of the thermolysin peptides at pH 6.5 (Offord [11]).

chemical structure during treatment with mild alkaline conditions (*N*-ethylmorpholine solution) should be hydrolysis of the aspartyl phosphate band, converting this group, which is negatively charged at pH 2, to an aspartyl residue, which bears only a partial negative charge at pH 2. Peptides undergoing this conversion should therefore possess higher mobilities towards the cathode at pH 2, and peptides T1a and T2a fulfil this criterion. In the case of the peptic phosphopeptide, a different approach was made, based on the knowledge that a cysteine residue was present, and the diagonal procedure of Brown and Hartley [10] was used, without loss of much phosphorus from the peptide. In this case, the mobility at pH 2.0 was greatly reduced, as expected from the formation of the additional negative charge of a cysteic acid residue.

Despite the use of favourable conditions, the gradual hydrolysis of the peptide necessitated the performance of several experiments before isolation of the pure tryptic peptide in sufficient yield for an accurate analysis was achieved.

This work confirms the tentative tripeptide sequence, (Ser or Thr)–Asp–Lys, predicted by Bastide et al. [2] on the basis of changes in electrophoretic mobility of the limit pronase peptide after various chemical modifications. It will be interesting to discover if the similarity in peptide structure of the active-site regions of the $[Ca^{++}]$-ATPase and the $[Na^+, K^+]$-ATPase [2] extends over a larger part of the polypeptide chain, as this would have implications for the evolution and mechanisms of the ion-translocating ATPases. It may also be significant that blockage of one of the sulphydryl groups of the ATPase inactivities it [12] and that substitution of a uniquely reactive lysine residue with pyridoxal phosphate also leads to inactivation [13]. However, it has yet to be proved that the residues responsible are the cysteine and lysine on either side of the aspartyl phosphate, nor is it known whether they are directly involved in the process of phosphate transfer.

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